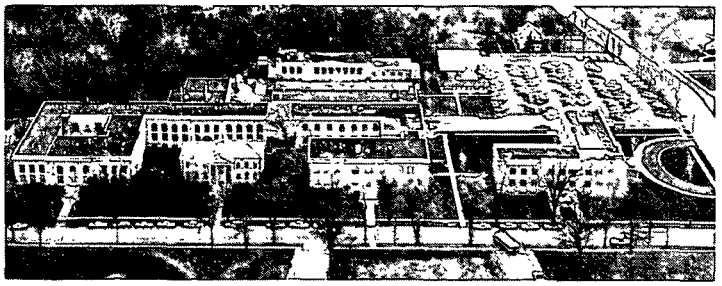

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USE OF MODEL SYSTEMS IN GYMNOSPERM EMBRYOGENESIS

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by

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ABSTRACT

The cell development and growth patterns of natural gymnosperm embryogenesis are presented. Comparisons with somatic embryogenesis in the wild carrot tissue culture system follow, from which generalized conclusions are made on the similarities between the two systems and the necessary developmental steps in embryogenetic systems.

[1]The authors are members of The Institute of Paper Chemistry's conifer tissue culture research team. Additional members of this team include S. Verhagen, J. Carlson, R. Feirer, G. Mignon, and K. Weyrauch. The authors wish to acknowledge that Dr. Donald Durzan was project leader when some of the reported research was being conducted.

INTRODUCTION

This is the first in a series of articles that will communicate to the Institute's member companies our approach and progress in attempting to obtain somatic embryogenesis in gymnosperm cultures. The research is part of the Institute's conifer tissue culture research program*.

BACKGROUND

The pulp and paper industry is intimately linked to the tree for its source of papermaking fibers, especially to those tree species called gymnosperms or conifers. Because of pressures on our industry and forest resources, it is important to obtain greater yields of fibers from our forests. A possible method of obtaining this improvement is through reforestation of our lands with genetically superior trees. There are two components in this endeavor: (1) selecting or obtaining genetically superior trees, and (2) mass producing the genetically superior individuals for planting in our forests.

Tissue culture research at the Institute is concerned primarily with developing a methodology for the production of large numbers of genetically identical superior trees through a process called "somatic embryogenesis." The latter is one of three principal routes for vegetatively propagating individuals: (1) traditional horticultural methods of rooting and grafting, (2) a tissue culture approach called "organogenesis," and (3) the tissue culture approach called "somatic embryogenesis." With many softwoods, traditional horticultural approaches are not currently possible. Also, only immature sources have been successfully employed in "organogenesis,"

*Reported results are part of the Institute's Project 3223, The Mass Production of Conifer Hybrids.

thus limiting genetic gain and, as a result, offering no significant advantage over the seed orchards approach.

In the process of successful somatic embryogenesis, a segment of a plant is excised and grown aseptically to produce a mass of cells called a callus. This callus is then placed in a liquid nutrient solution, and free single cells are sloughed off into the liquid medium. These single cells are further manipulated to produce "somatic embryos," which are similar to natural seed embryos in that both can be grown into a mature plant. Thus, through the process of "somatic embryogenesis" it is feasible to produce millions of identical copies of an individual. Also, since we are growing an individual from a single cell, the possibility of "genetically engineering" a new or superior individual exists. Although there has as yet been no reported success in obtaining somatic embryogenesis in gymnosperms, other plant species have been successfully cultured.

In our attempts to obtain somatic embryogenesis in gymnosperms, it has became very obvious that traditional tissue culture techniques and media would not be successful and that additional data and hypotheses would be needed. The following is the first in a series of reports that will summarize our current understanding, speculations, and ideas concerning natural and somatic embryogenesis in gymnosperms. As additional experiments are conducted and our understanding develops, so will our perception of our system.

HYPOTHESIS AND MODEL SYSTEMS

Our primary hypothesis is that natural and somatic embryogenesis are governed by the same laws of nature, and the better we understand natural embryogenesis the greater will be our chances of success in somatic embryogenesis. Many studies have been conducted on the development of both animal and plant embryos. However, for plants,

previous studies were concerned primarily with detailing the specific cell division patterns. There have been no significant studies that attempt to interpret natural gymnosperm embryo development in terms of its implications and correlations with gymnosperm tissue culture techniques and hypotheses.

Our intention is to try to understand the sequence of events that occur in nature, so that this information can be applied to our attempts to achieve somatic embryogenesis.

NATURAL GYMNOSPERM EMBRYOGENESIS

The immature seed immediately prior to fertilization is composed of three distinct and important zones: (1) the archegonium or egg cell, (2) the erosion zone or erosion cavity, and (3) the gametophytic tissue. These zones are illustrated in Fig. 1.

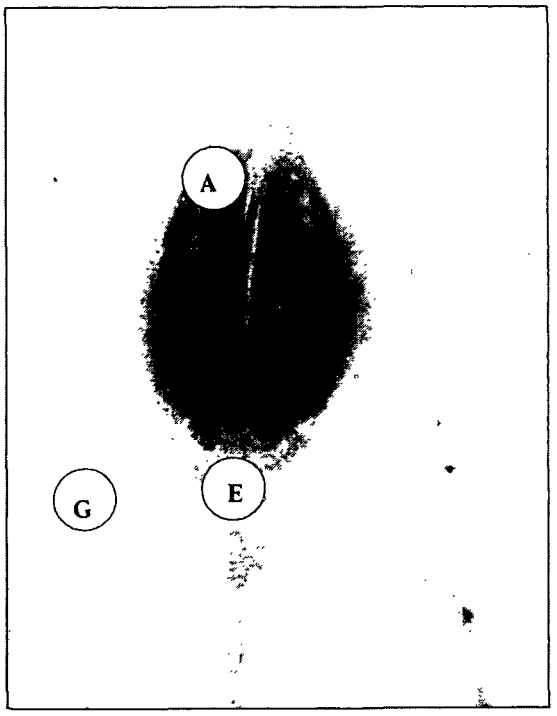

Figure 1. Zones present in an immature Douglas-fir seed immediately prior to fertilization, A = Archegonium, E = Erosion Zone, G = Gametophytic Tissue. (50X)

Basically, natural gymnosperm embryogenesis can be divided into three phases corresponding to the three environments or growth media that the embryo will encounter during development. The sequence of events is as follows:

Phase 1 - Initiation - Fertilization occurs in the archegonium; following fertilization, a three-tiered, 12-celled proembryo is formed.

Phase 2 - Transition/Build-up - The proembryo emerges from the archegonium and enters the environment of the erosion zone. As hydrolytic enzymes apparently digest the erosion zone, the proembryo traverses downward through the erosion zone (Fig. 2). It normally takes 2-3 weeks for the embryo to reach the bottom of the erosion zone. During this time the proembryo increases in size from 12 cells to an early embryo of only 40 or so cells (Fig. 3).

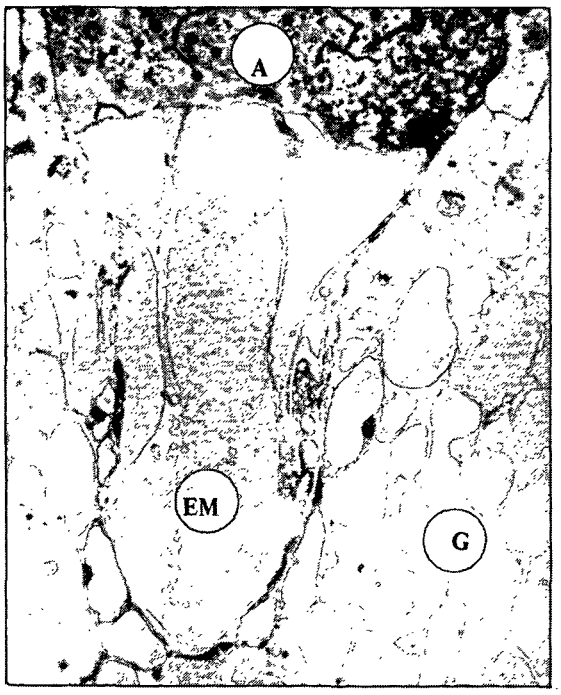

Figure 2. Proembryo emerging from archegonium and starting to traverse downward into the erosion zone, EM = Embryo, A = Archegonium, G = Gametophytic tissue. (350X)

Figure 3. Early embryo as it appears immediately prior to being launched. (400X)

Phase 3 - Development - This is the most significant phase in natural embryogenesis and the phase that must be understood if successful somatic embryogenesis is to be obtained. As the 40-celled early embryo moves from the erosion zone into the gametophytic zone, it (1) enters a more dilute high moisture content region, and (2) there is an influx of nutrients into the seed. This influx of material so alters the environment that wherever the influx occurs, there is immediate reaction or "blooming" of the gametophytic cells (Fig. 4). Through the action of one or both of these changes the embryo itself is launched, in that it accelerates in terms of growth rate,

and differentiates or polarizes into a mature embryo. The rate of lipid synthesis is also maximal at this time (Fig. 5).

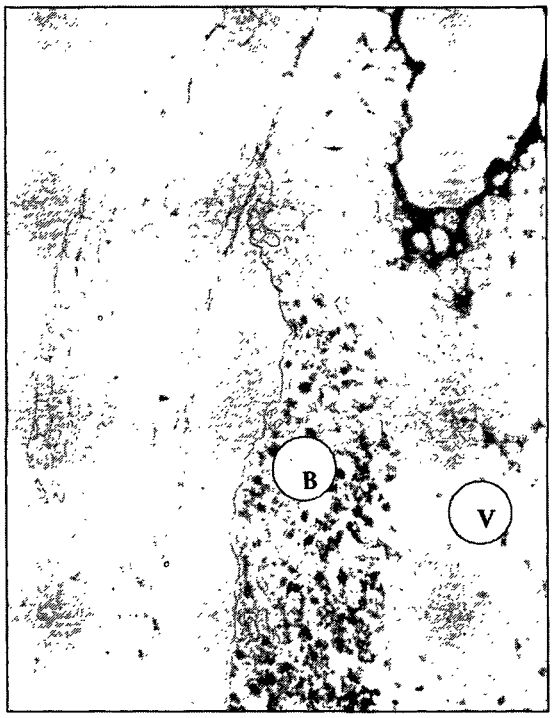

Figure 4. Gametophytic cells "blooming" with influx of nutrients into seed, B = Blooming Gametophytic Cells, V = Vacuolated Gametophytic Cells. (100X)

The time sequence for the above events and a growth curve for the developing embryo are shown in Fig. 6 and 7. Most gymnosperms follow the same sequence of events and the same timetable between fertilization and mature embryo development.

WILD CARROT SOMATIC EMBRYOGENESIS

The wild carrot somatic cell system is one prototype of the system we are trying to develop for gymnosperms. We have maintained an embryogenic line of wild carrot to help develop concepts and models. Also, there is a considerable amount of data in the literature concerning this system, thus making it a very desirable model

system. The brief explanation of the wild carrot system that follows will allow comparisons to be made between this system and natural gymnosperm embryo development.

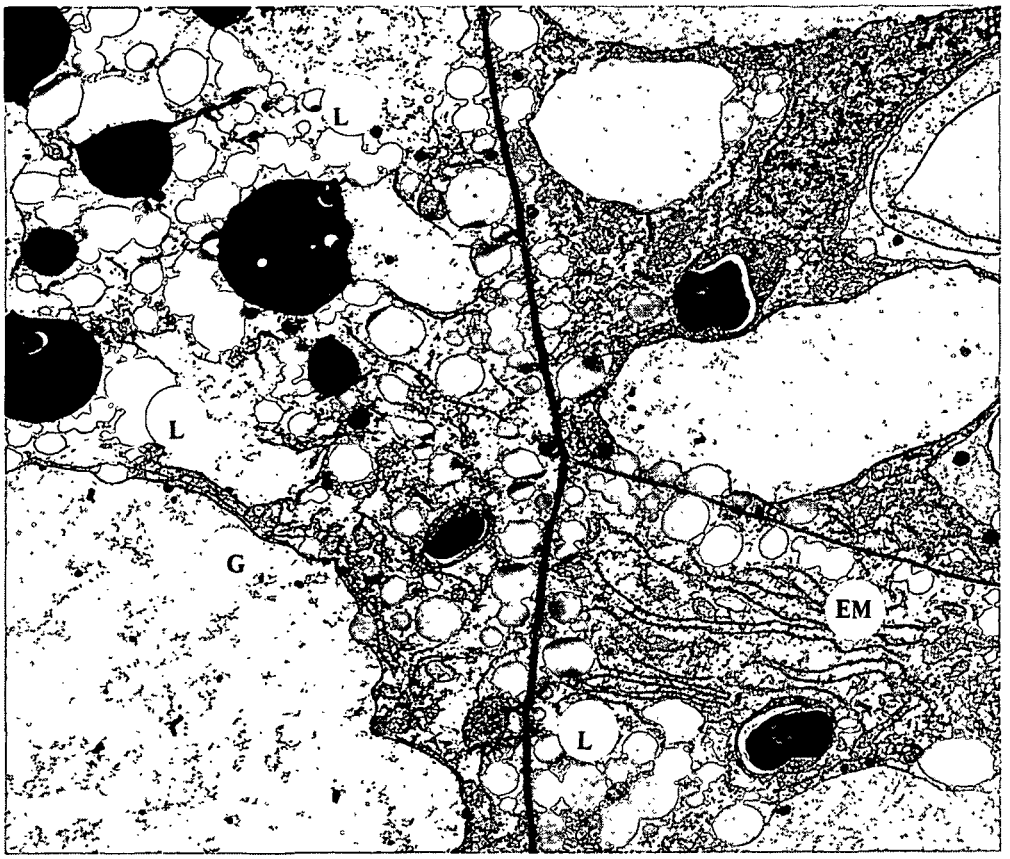

Figure 5. T.E.M. micrograph of embryo at launch point showing appearance of lipids at this stage of development. EM = Embryo, G = Gametophytic Tissue, L = Lipids. (3500X)

The wild carrot system depends upon taking an initial explant and obtaining calli from the explant. The calli are grown on agar with an auxin (2,4-D) and a specifically designed wild carrot (W.C.) medium. Once the calli are obtained, they are suspended in a liquid W.C. medium with auxin as a growth regulator. At this point the cells can be maintained as a cell suspension indefinitely. This is accomplished by periodically (approximately every two weeks) subculturing the flask contents.

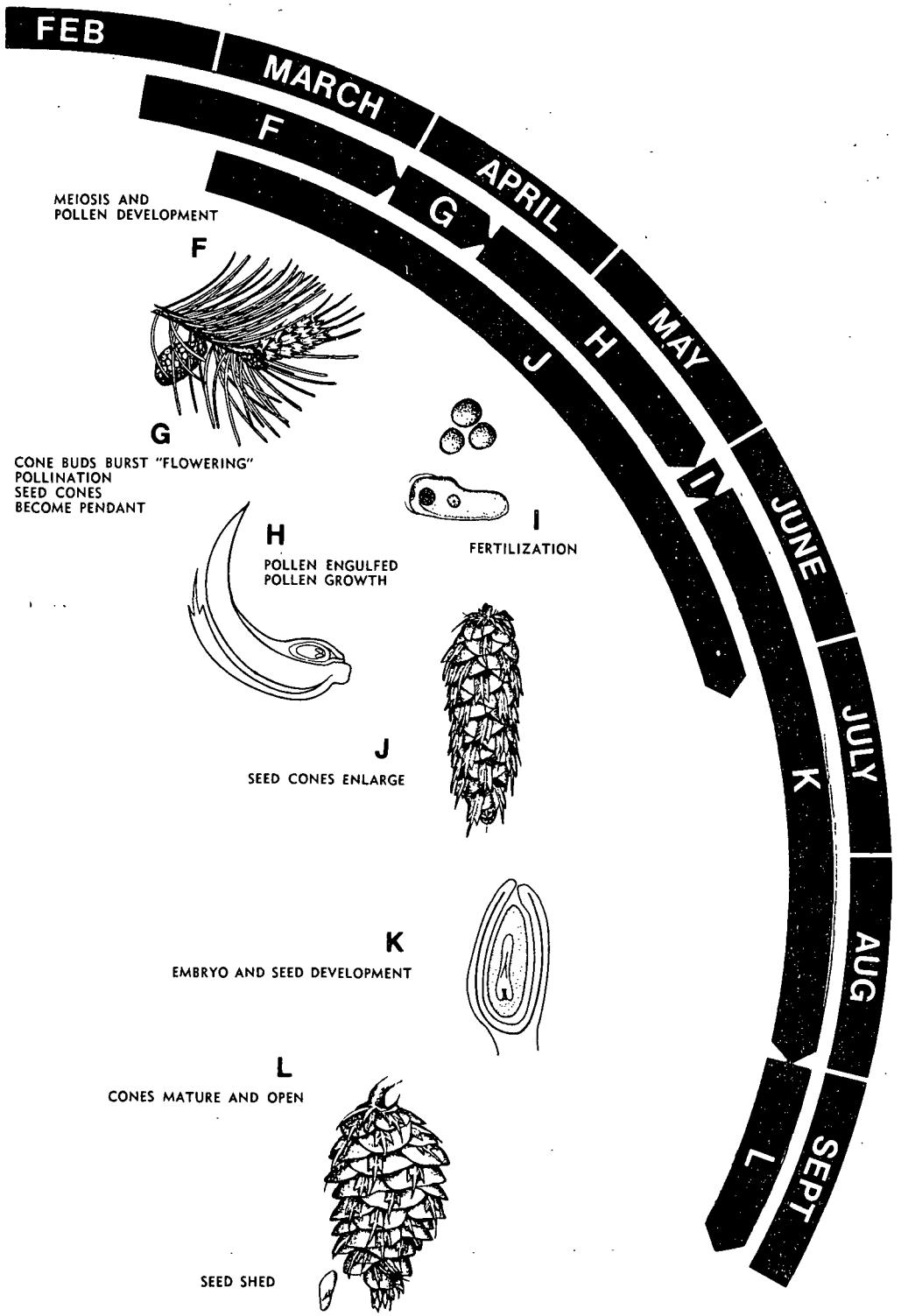

Figure 6. Seasonal time sequence of seed development, from "The Life History of Douglas-fir" by George S. Allen and John N. Owens, Information Canada, Ottawa, 1972.

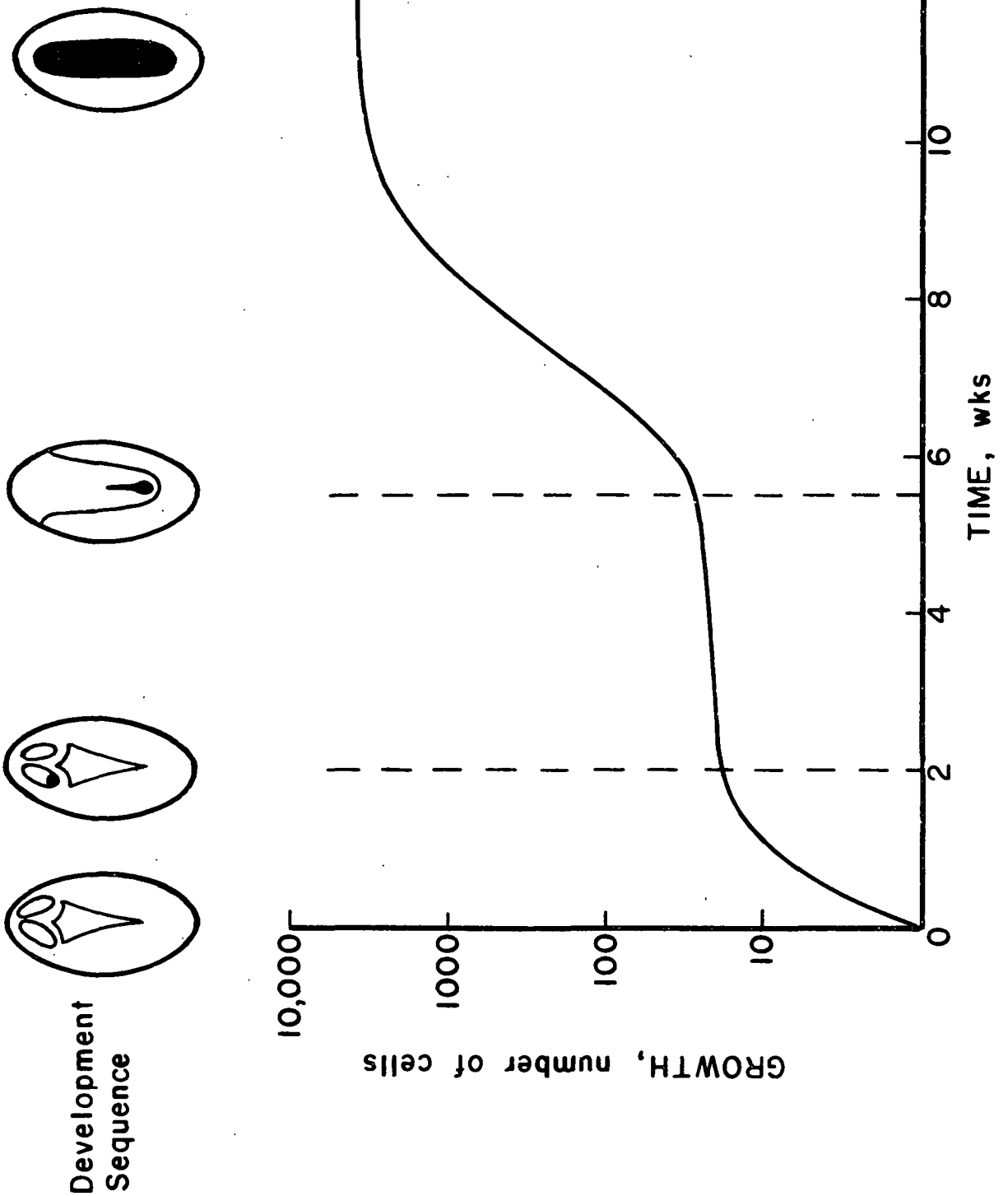

Figure 7. Developmental sequence and growth curve for natural Douglas-fir embryogenesis

If somatic embryos are desired, the liquid cell suspension is screened to obtain the required size or fraction of clumps (proembryonic masses, PEM's) which are placed in fresh liquid medium that contains no growth regulator. Placing the PEM's into the medium without growth regulator launches the clumps into a series of developmental steps culminating in a somatic embryo capable of producing an intact plant. A growth curve for such a series of events is shown in Fig. 8.

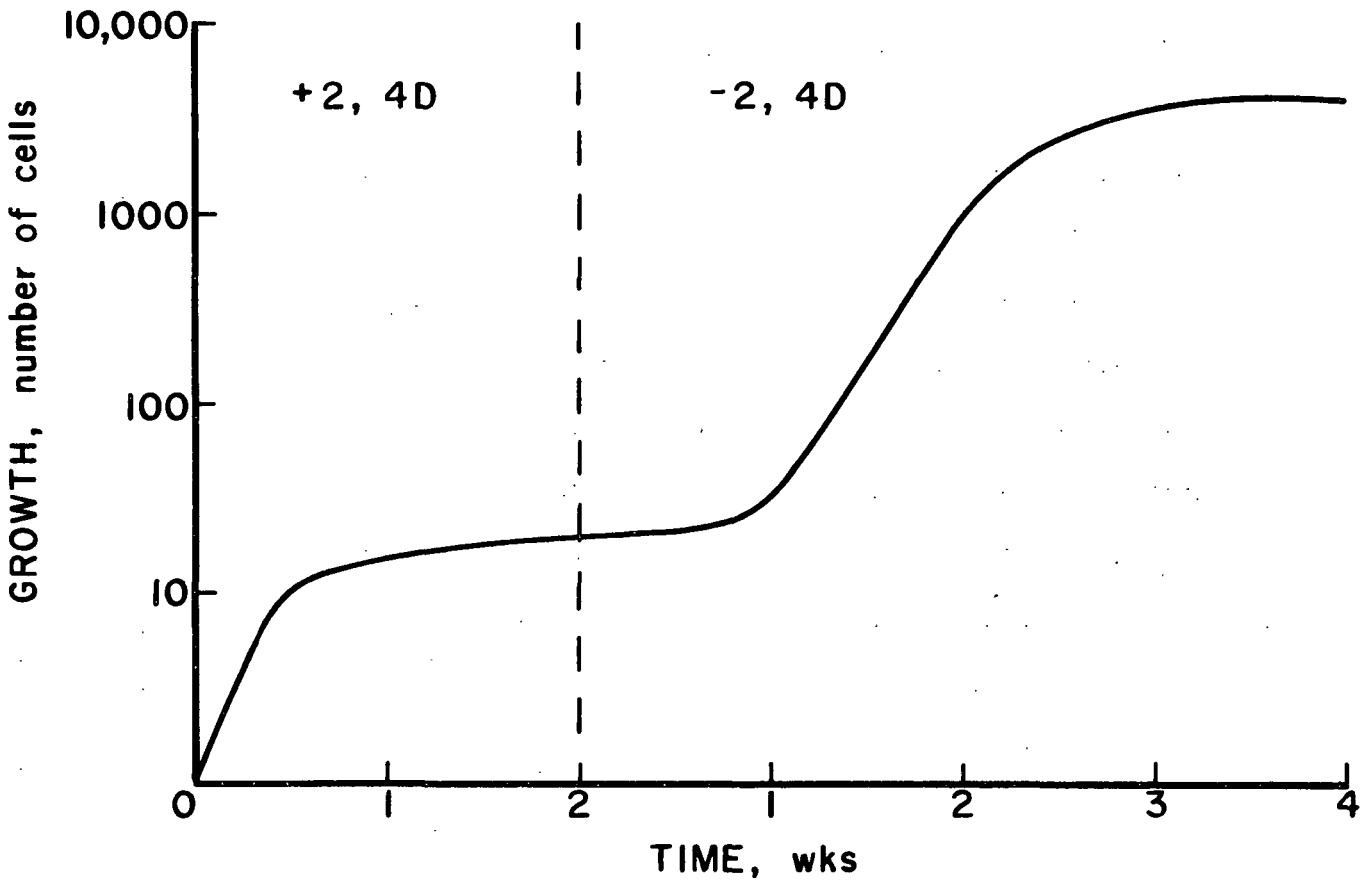

Figure 8. Wild carrot somatic embryogenesis growth curve

SIMILARITIES BETWEEN NATURAL AND SOMATIC EMBRYOGENESIS

Observations made on wild carrot somatic embryogenesis and natural embryogenesis of Douglas-fir and loblolly pine indicate that natural and somatic embryogenesis have the following points in common:

1) The growth curves for both systems are composed of two sigmoid curves.
2) The first sigmoid curve results in a preformed clump or proembryonic mass (PEM).
3) The clump which is launched toward embryogenesis in both cases is at least three cell diameters wide or contains approximately 40 cells.
4) During the second growth curve the preformed clump (PEM) develops into a mature embryo exhibiting both roots and shoots.
5) The second growth curve is orders of magnitude larger than the first growth curve (see Fig. 8).
6) The polarized growth obtained in the second growth curve is preceded by an abrupt change in the PEM environment. In the wild carrot system this change is caused by removing the synthetic growth regulator, whereas in the natural system dilution and an influx of nutrients alter the environment.
7) The response of the embryo to the change in environment is very rapid. Most importantly there is rapid increase in cell divisions, specifically asymmetric internal divisions, which result in compartmentalization and a reduction in average cell size (Fig. 9).
8) The ultrastructure of the meristematic cells of the carrot and conifer systems is very comparable histologically.

A.

B.

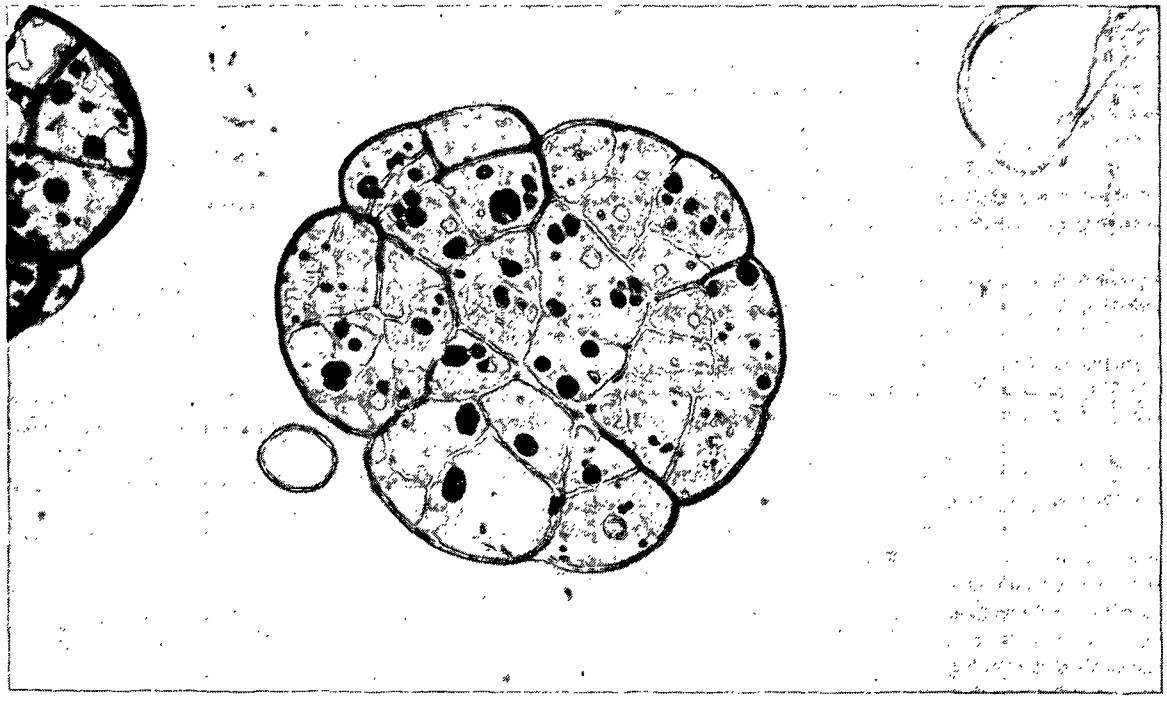

Figure 9. Natural Douglas-fir (A) and somatic wild carrot PEMS (B) immediately after launch, both at 1000X

9) Both systems work under comparable physical constraints (i.e., in the dark at ambient temperature and while shaken).

The implications of the above similarities between the two systems are that they are controlled, for the most part, by the same environmental/nutritive/kinetic factors.

An overly simplified scheme or explanation of embryogenesis in general would be that embryogenesis is obtained by growing a single cell into a small clump [at least three cell diameters (5) wide]. Then, through manipulation of the media and growth regulators, a gradient or stress arises in the clump that causes it to rapidly develop through cell division and differentiation into an organized structure.

FUTURE

Based on our hypothesis and the model systems, we defined two objectives of the current project.

Objective I - To obtain and maintain a suspension of gymnosperm PEM's.

Objective II - To manipulate gymnosperm PEM's to produce somatic embryos and, ultimately, mature plants.

Future publications dealing with our conifer tissue culture research are expected to fall into one of three categories:

(1) research on the model systems,

(2) research directed at achieving Objective I, and

(3) research directed at achieving Objective II.